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SYMPOSIUM

Manipulation of Primary Sex Ratio in Birds: Lessons from the Homing Pigeon (*Columba livia domestica*)

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Synopsis Across various animal taxa not only the secondary sex ratio but also the primary sex ratio (at conception) shows significant deviations from the expected equal proportions of sons and daughters. Birds are especially intriguing to study this phenomenon as avian females are the heterogametic sex (ZW); therefore sex determination might be under direct control of the mother. Avian sex ratios vary in relation to environmental or maternal condition, which can also affect the production of maternal steroids that in turn are involved in reproduction and accumulate in the developing follicle before meiosis. As the proximate mechanisms underlying biased primary sex ratio are largely elusive, we explored how, and to what extent, maternal steroid hormones may be involved in affecting primary or secondary sex ratio in clutches of various species of pigeons. First we demonstrated a clear case of seasonal change in sex ratio in first eggs both in the Rock Pigeon (*Columba livia*) and in a related species, the Wood Pigeon (*Columba palumbus)*, both producing clutches of two eggs. In the Homing Pigeon (*Columba livia domestica*), domesticated from the Rock Pigeon, testosterone treatment of breeding females induced a clear male bias, while corticosterone induced a female bias in first eggs and we argue that this is in line with sex allocation theory. We next analyzed treatment effects on follicle formation, yolk mass, and yolk hormones, the latter both pre- and post-ovulatory, in order to test a diversity of potential mechanisms related to both primary and secondary sex ratio manipulation. We conclude that maternal plasma hormone levels may affect several pre-ovulatory mechanisms affecting primary sex ratio, whereas egg hormones are probably involved in secondary sex ratio manipulation only.

Introduction

According to Mendelian laws and given genetic sex determination, the chance to produce a son or a daughter should be equal, but a large number of studies show that the sex ratio of offspring, defined as the proportion of sons, can significantly deviate from the expected 50% (see reviews for mammals: Grant and Chamley 2010; reptiles: Radder et al. 2007; birds: Pike and Petrie 2003, Alonso-Alvarez 2006, Goerlich and Groothuis, in preparation). Several hypotheses exist as to why the sex ratio of offspring should be biased, e.g., if in certain environments the reproductive fitness varies between the sexes, parents should overproduce the sex with the greater fitness returns (Trivers and Willard 1973). However, in order to really understand the consequences on fitness, selective advantages, and potential trade-offs, we need to identify the proximate mechanism of the manipulation of offspring sex ratio. Manipulation of the primary sex ratio, i.e., the proportion of sons at conception, is especially intriguing as influencing sex determination would spare the costs of producing offspring of the undesired sex. However, despite increasing evidence for biased primary sex ratios, the underlying mechanism remains largely elusive.

Birds offer intriguing possibilities for studying manipulation of the primary sex ratio, as avian females are the heterogametic sex and therefore possibly in control of the sex of their offspring. Several potential mechanisms have been proposed to explain the

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phenomenon of biased sex ratio in birds (reviewed by Pike and Petrie 2003; Alonso-Alvarez 2006) and we have investigated these in our experiments on the Homing Pigeon (*Columba livia domestica*) as reviewed in this article.

We summarize our experiments on the Homing Pigeon in which we tested mechanisms that bias primary sex ratio. We manipulated female hormones and body mass and measured yolk mass, egg mass, yolk hormones, fertility rate, and oviposition patterns. We believe that studying the mechanisms of adjusting primary sex ratio through using different approaches and techniques in a single species provides a comprehensive picture of ongoing reproductive processes. This facilitates detection of new patterns and details that can shed light on the underlying mechanisms for the sex ratio of offspring and its manipulation by parents. The results provide starting points to be tested in other species as well, although one should keep in mind that different species might have developed different mechanisms, depending on reproductive physiology and life history.

Potential timing of manipulation of primary sex ratio

Manipulation of the primary sex ratio could occur at several stages in the development of the ovum and the following mechanisms have been suggested in the literature. (1) Asynchronous follicle development: Instead of maturing in a strictly hierarchical manner, pre-ovulatory follicles might develop in a sex-specific way, which could explain the sex-biased oviposition patterns often observed in birds (Ankney 1982). This could mean that a certain factor, possibly in combination with differential rate of follicular maturation, might determine the outcome of sex determination at meiosis. Alternatively, Badyaev et al. (2006) have suggested that follicles develop in (sex-specific) clusters, which could lead to differential exposure to the maternal environment and therefore to differential accumulation of maternally derived substances such as hormones or carotenoids, which in turn might influence the outcome of meiosis. Thus, both these hypotheses rely on non-random chromosome segregation during meiosis, which is at the moment the most supported potential mechanism that results in a skewed primary sex ratio. (2) Meiotic drive (non-random chromosome segregation): The oocyte undergoes meiosis I just prior to ovulation. During meiosis I the diploid sets of chromosomes are split and the developing, haploid ovum retains either the Z (male) or W (female) sex chromosome, while the other is excluded to the inactive polar body. Substances in the yolk, accumulated during the rapid-growth phase, could influence meiosis to produce a particular sex (see above; Badyaev et al. 2008). Alternatively, follicles are highly vascularized and the composition of maternal plasma might influence segregation of chromosomes such that the preferred sex chromosome is retained in the ovum (Oddie 1998; Rutkowska and Badyaev 2008). (3) Follicular abortion: If after meiosis the ovum contains the non-preferred sex chromosome, it could be aborted instead of being ovulated (Emlen 1997). This could occur via ovulation into the peritoneal cavity, although this might lead to health issues and is considered rather a pathological phenomenon. A more common process is follicular atresia, i.e., the death and re-absorption of follicles (Johnson and Woods 2007). (4) Sex-specific fertilization/zygote development: This mechanism is, strictly speaking, a post-ovulatory, secondary mechanism determining offspring sex ratio. However, as eggs are usually (artificially) incubated for several hours to days in order to get sufficient embryo tissue for molecular sex determination (but see Aslam et al. 2012), eggs without visible embryonic development might point to adjustment of sex ratio via selective fertilization/zygote development.

The pigeon as a study species

Aristotle was fascinated by the sex ratio of pigeons' offspring and stated: "the pigeon, as a rule, lays a male and a female egg, and generally lays the male egg first" (Aristotle *ca.* 350 BC). He may have made his observations in spring as in two pigeon species studied in the field (Wood Pigeon, *Columba palumbus*) and under semi-natural conditions (Feral Pigeon, *Columba livia*, the wild ancestor of the domesticated species), respectively, first eggs, but not second eggs, show a distinct seasonal shift in primary sex ratio with an overproduction of sons in the beginning, and an overproduction of daughters late in the season (Dijkstra et al. 2010). This seasonal shift stimulated the study, not only of its functional consequences (males may require a longer time span to become sexually mature) (Dijkstra et al. 1990; Goerlich et al., in preparation) but also of the proximate mechanisms (Goerlich et al. 2009, 2010a, 2010c). In our further experimental approaches we worked with homing pigeons (*Columba livia domestica*) because this domesticated strain is much more convenient for laboratory experiments than is its wild ancestor.

For several reasons, homing pigeons are especially suitable to study the mechanisms underlying the manipulation of the sex of offspring and the allocation of

yolk hormones. The birds of the family *Columbidae* are considered to be determinate layers and produce a modal clutch size of two eggs, laid at a consistent interval of ~44 h (homing pigeons: Levi 1945; Holmberg and Klint 1986; Johnston and Janiga 1995). The two follicles are estimated to accumulate yolk for about 6.5 until the mature state is reached and ovulation occurs (Birrenkott et al. 1988). The passage through the oviduct lasts about 44 h, during which the ovum is fertilized and subsequently albumen and shell are formed. Shortly after the first egg is laid, the second follicle is ovulated (Levi 1945). The small clutch size has the advantage that any bias in sex ratio of the offspring is relatively easy to detect. Since clutch size and the interval between eggs are highly consistent, any deviation might serve as an indicator that manipulation of the primary sex ratio has occurred. Finally, Pigeons readily breed in captivity nearly year round and are sufficiently large that it is possible to repeatedly collect blood for measurement of hormone levels at different stages of egg-production; such information is important for exploring the effects of maternal hormones.

After 18 days of incubation, the chick (squab) from the first egg is usually the first to hatch (Johnston and Janiga 1995; Goerlich et al., unpublished data). The parents immediately feed the newly hatched squabs with ''pigeon milk,'' a substance formed by the crop sack of both parents; this feature is unique to Columbidae (Johnston and Janiga 1995). The high energy content of the crop milk results in fast growth of the young during early days. Prolonged intervals between hatching of the first and second chick usually results in the death of the last young to hatch (Johnston and Janiga 1995; Goerlich et al., unpublished data). This detrimental consequence might be the functional explanation of the consistency of the interval between oviposition of the first and second egg. Thus, mechanisms of sex-ratio manipulation that would result in delay of oviposition and/or hatching of the second chick are rather unlikely to exist in pigeons.

Manipulation of primary sex ratio by homing pigeons and its underlying mechanisms

Manipulation of maternal hormones

In several experiments we manipulated the hormonal state of breeding females to investigate the effects of maternal steroid hormones in plasma and yolk on sex ratio of the offspring. While studies on the effects of testosterone manipulation on primary sex ratio and female reproductive physiology have been published elsewhere (Goerlich et al. 2009, 2010a, 2010b, 2010c), we will report our findings on the effects of an elevation of maternal corticosterone in this article.

In order to rule out indirect effects of the hormone treatment on offspring's sex ratio induced by social interactions (Veiga et al. 2004), we kept breeding pairs in separate cages during our experiments. The techniques of hormone manipulation were carefully tested by pilot experiments to ensure a long-term elevation within the physiological range (testosterone: Goerlich et al., unpublished data; corticosterone: Goerlich 2009). We chose to elevate testosterone by implanting females with hormone-filled Silastic tubes, a common method that is successful in achieving stable elevated plasma levels while avoiding possible confounding effects induced by repeated injections.

During the experiments eggs were collected within 30 min after oviposition and the sex of embryos was determined after 3 days of artificial incubation, the time required to obtain sufficient samples of DNA for sexing (Griffiths et al. 1998).

Since none of our treatments decreased egg fertility, we are confident that any bias present in the primary sex ratio did not come about via post-ovulatory mechanisms such as sex-specific fertilization or mortality, but rather was induced prior to fertilization, thereby being indicative of the primary and not the secondary sex ratio.

As in the previous experiments on pigeons (effect of food manipulation: Pike 2005; seasonal changes: Dijkstra et al. 2010), hormone treatment affected only the sex of the first egg. Testosterone treatment induced a male bias (Goerlich et al. 2009, but see Goerlich et al. 2010c), in line with similar earlier studies on testosterone (Veiga et al. 2004; Rutkowska and Cichon 2006; but see Pike and Petrie 2006). Even more intriguing was the fact that in our experiment treatment of the mothers with testosterone for 3 weeks induced a male bias not only in the clutch produced during the treatment but also in the clutch laid almost 1 year later. Since birds were kept isolated in pairs, the order in the social rank among females cannot have been relevant and the data suggest long-term effects of the treatment on the physiology of the ovaries (Goerlich et al. 2009).

To test the effects of elevated plasma corticosterone on primary sex ratio, we formed breeding pairs of homing pigeons ($N = 39$) and treated the females with either of three different methods: Subcutaneous implants of pellets (Innovative Research, Sarasota FL, USA), implants made from hardened palm fat containing corticosterone (Pickering and Duston 1983), or food mixed with 1 ml of crystalline corticosterone

dissolved in vegetable oil. Each of these groups was further split into a control subgroup and three additional subgroups of increasing dosage of corticosterone (low, intermediate, and high) (Goerlich 2009). Females were treated for 4 weeks during which eggs were removed after completion of the clutch and artificially incubated for 3 days after which the sex of the embryo was determined. Subsequent eggs were treated the same way. We measured body mass and tarsus of females on the day that treatment began and measured body mass four additional times over the course of the experiment to calculate maternal condition (see below).

For each group, we performed a linear mixed-effects regression to test the effect of dosage on the multiple corticosterone measurements obtained from each bird and included dosage as fixed factor and individual ID as random factor. We found a strong positive association between dosagae of corticosterone and concentrations of plasma corticosterone in all three groups (fat implants $t = 3.443$, $P = 0.001$; food $t = 2.908$, $P = 0.004$; pellets $t = 5.540$, $P < 0.001$), so we used the dosage variable to rank the concentrations of hormone treatment for all treatment methods (control = 0, low = 1, intermediate = 2, high = 3).

To test the effect of corticosterone dosage on the embryos' sex we performed a logistic mixed-effects regression and included method of treatment, clutch number, and individual ID as random factors. We found a significant interaction between dosage category and position in the laying sequence (egg order) in predicting sex ratio (Fig. 1; Table 1a) and the effect was similar for all treatments ($t = -0.005$, $P = 0.99$). In *post hoc* tests in which first and second eggs were analyzed separately, first eggs showed an increasing bias towards female embryos with increasing corticosterone dosage (Table 1b) but second eggs did not (Table 1c). This differential effect for first and second eggs is in line with all our other studies in which only the sex of the first egg was affected.

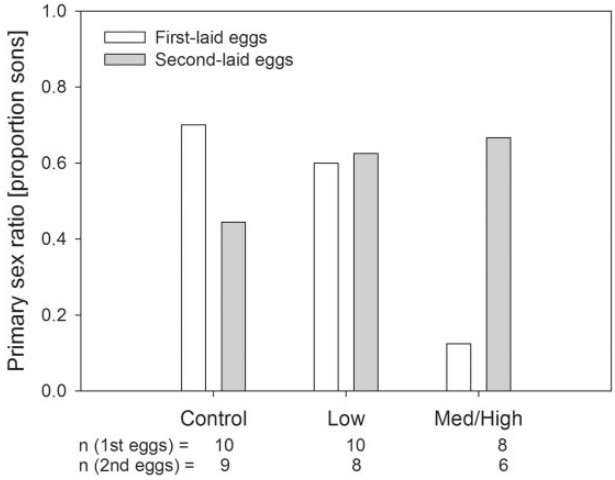

Fig. 1 Sex ratio for first eggs and second eggs separately as a function of corticosterone dosage. Dosage is ranked on the x-axis into three increasing categories of controls, low dosage and the last group combines birds that received intermediate and high dosages. Sample sizes are shown below the x-axis.

Blood samples were taken from all birds 11 times during the experiment to establish individual plasma corticosterone profiles. Graphs of corticosterone profiles were created using SigmaPlot and exported as jpegs into ImageJ (freeware produced by NIH: http://rsb.info.nih.gov/ij/) with which we measured the area under the curve within the 2 days before the egg was laid, when meiosis occurs (Olsen and Fraps 1950; Johnston and Janiga 1995). The resulting corticosterone-exposure variable was log-transformed to meet normal distribution. Again, using a logistic mixed-effects regression, with treatment method, clutch number, and individual ID as random factors, we tested the interaction between corticosterone exposure during meiosis, maternal body condition, and egg order as a fixed factor. We found no evidence for a relationship between sex ratio and maternal condition during the 2 days before oviposition as all predictors, including body condition, were excluded from the final model (Table 2). We did, however, find that exposure to maternal corticosterone shortly before oviposition interacted significantly with egg order to predict the sex of the embryo (Table 2), in congruence with the significant interaction between corticosterone dosage and egg order in predicting sex ratio. Our results are in line with previous studies on the effects of a chronic elevation of corticosterone (Pike and Petrie 2006; Bonier et al. 2007; but see Love et al. 2005).

Table 1 Mixed-effects logistic regression of the effects of corticosterone treatment on sex ratio

Predictors	*B*	SEM	*t*-value	*p*
(a) All eggs				
Dosage (control, low, medium, high)	−3.06	1.25	−2.45	0.014
Egg order (first egg, second egg)	−1.49	0.92	−1.61	0.107
Dosage*egg order	1.84	0.78	2.37	**0.018**
(b) First eggs only				
Dosage	−1.27	0.59	−2.17	**0.03**
(c) Second eggs only				
Dosage	0.76	0.58	1.31	0.19

Random factors include treatment method, clutch number, and individual. Results of significant predictors are indicated in bold.

Table 2 Backwards step-wise mixed-effects logistic regression testing the relation between body condition and the sex of embryos in eggs

	Final model				Before exclusion	
Predictors	***B***	**SEM**	***t*-value**	***P***	***t*-value**	***P***
Cort	−1.54	0.80	−1.92	0.055		
Egg order	−1.17	0.87	−1.35	0.177		
Cort*egg order	1.03	0.53	1.96	**0.0497**		
(1) Cort*body cond*egg order					−0.36	0.722
(2) Body cond*egg order					−0.94	0.346
(2) Cort*body cond					1.48	0.139
(4) Body cond					0.59	0.550

Random factors included are treatment method, clutch number, and individual ID. Numbered predictors indicate order of exclusion from the model. Cort refers to exposure to corticosterone before oviposition, egg order refers to position in the laying sequence (1 or 2), body cond refers to residual body mass before oviposition.

A mixed-effects regression on only first eggs indicated that corticosterone dosage significantly delayed oviposition of the first egg ($t = 2.63$, $P = 0.015$). However, we found no effect of the sex of the embryo ($t = -0.262$, $P = 0.796$) or any interaction between embryos' sex and dosage ($t = 1.512$, $P = 0.144$) in predicting the initiation of clutches. As expected, given the lack of any bias in sex ratio in second eggs, we found no effect of corticosterone dosage on the time interval between oviposition of first and second eggs (mixed-effects regression: $t = -1.122$, $P = 0.275$). We also found no evidence for premature ovulation of second follicles in corticosterone-treated females because the yolk weights of second eggs were not smaller than those of second eggs from control females (egg order * dosage: $t = 1.077$, $P = 0.285$; dosage effect in first eggs: $t = 1.383$, $P = 0.172$; dosage effect in second eggs: $t = 1.364$, $P = 0.187$).

In conclusion, treatment of mothers with testosterone induced a lasting male bias and treatment with corticosterone induced a female bias. The bias was apparent in first eggs only and we found no evidence for processes other than meiotic drive (see also below).

Steroids or body mass?

While performing hormonal manipulations we repeatedly weighed females to further investigate the importance of maternal body condition, a factor often shown to influence primary sex ratio (Pike and Petrie 2003; Alonso-Alvarez 2006). Studies on birds often use body mass corrected for structural size as a proxy for body condition (in our case the residuals of a regression of mass on tarsus size). Mothers in good body condition are predicted to bias their primary sex ratio toward the sex that gains most from a high quality background (Trivers and Willard 1973); this is usually the male sex, as in males reproductive success strongly depends on the ability to attract females. Importantly, both testosterone and corticosterone often are found to be correlated with body condition; usually high testosterone levels indicate a good physiological condition, while high corticosterone levels indicate a low physiological condition (Kitaysky et al. 1999; Perez-Rodriguez et al. 2006). Indeed, both elevation of corticosterone and a low body condition have been found to be related to an overproduction of daughters (Pike and Petrie 2005). In our experiment, the correlation between corticosterone and body mass was uncoupled (see above). The corticosterone treatment plus the amount of food being provided *ad lib* abolished the natural negative correlation between corticosterone and body condition and corticosterone-treated females actually increased in body mass. We found that plasma levels of corticosterone at about the estimated time of meiosis of the first egg predicted the embryo's sex while body condition had no significant effect. This suggests that in nature (without *ad lib* food) a low body mass stimulates the production of corticosterone and thereby affects offspring's sex ratio.

When manipulating testosterone, we did not find an effect of the treatment on maternal condition and no effect of condition on primary sex ratio; only the main effect of the treatment induced a male bias (Goerlich et al. 2009). We then designed a follow-up experiment to further disentangle the influence of testosterone, corticosterone, and indices of body mass on primary sex ratio (Goerlich et al. 2010c). We repeated the testosterone experiment and implanted females with the same dosage as before, but additionally measured corticosterone, cholesterol, and glucose, all indicators of body condition. In this experiment, some of the females changed considerably in body mass, due to a difference in housing conditions before and during the experiment. Surprisingly, testosterone did not induce a bias in primary sex ratio relative to control females but females changed the sex composition of the first eggs of two subsequent clutches according to individual change in body mass, while absolute body mass (measured prior and after clutch completion) and

body condition (residual body mass) did not predict the sex of the offspring. Irrespective of treatment group, females that gained mass were more likely to produce a male in the final clutch while females that lost mass were more likely to produce a daughter, which indicates that the body-mass cue overruled the hormonal cue.

These results fit well with evolutionary predictions, namely that when sons are more costly to produce than daughters, as is likely the case in pigeons; males are about 15% heavier than females (Johnston and Janiga 1995), so mothers should overproduce sons when in good condition and daughters when in poor condition. It is clear that maternal body mass and/or condition should always be taken into account, and preferably not only the current state but also the change in body mass at about the time of conception (Cameron and Linklater 2007) or egg production (Goerlich et al. 2010c).

In the latter experiment we found a negative correlation between plasma testosterone and plasma corticosterone under un-manipulated conditions (Goerlich et al., unpublished data). However, testosterone implantation did not affect plasma levels of corticosterone, or the levels of glucose or cholesterol, despite testosterone levels being elevated (Goerlich et al. 2010c). Also, corticosterone levels did not predict primary sex ratio or the change in sex composition, as did the change in body mass (Goerlich et al. 2010c). Therefore it is still not completely resolved whether testosterone acted directly on the primary sex ratio in the first experiment (Goerlich et al. 2009). We are currently investigating whether corticosterone might affect sex determination via its influence on circulating levels of maternal testosterone.

To further study this complex interaction, circulating levels of corticosteroid-binding globulins (CBGs) should be measured as well. CBGs regulate the bioavailability of corticosterone (the major part of circulating corticosterone being bound to CBG and thus considered inactive) but show affinity to testosterone as well (Breuner and Orchinik 2002). As steroid assays usually measure the total amount of plasma hormones and do not discriminate between bound and free fractions, changes in unbound hormone levels might not be accurately measured.

The first egg effect and abortion of follicles

As mentioned above, all the five studies investigating manipulation of the primary sex ratio in pigeons showed that the sex of the first egg can be biased, but none found evidence that this holds true for the second egg. A stronger sex bias in the first egg relative to the second egg was explained by Pike (2005) on the basis of a *post-hoc* model of sex-specific abortion of follicles (following Emlen 1997). He proposed that the first follicle bearing the un-preferred sex chromosome could be aborted and replaced until a follicle carrying the preferred sex chromosome would be next in line in the pre-ovulatory hierarchy. This follicle would be ovulated and end up in the first egg. The next follicle in line, potentially forming the second egg, could also be aborted once, but then no further abortions would take place, so that the subsequent follicle in the hierarchy would always be ovulated regardless of sex. This model was invoked to explain the strong sex bias in first eggs and the much weaker but still significant effect in second eggs (Pike 2005). However, our recalculation yielded, as in our studies, no significant effect in the second egg so that we used the model only for the first egg.

Manipulation both of testosterone and corticosterone not only resulted in a bias of the sex of first eggs but also delayed initiation of the clutch. The sex of the second egg, however, was not affected and interval between eggs remained constant (see above; Goerlich et al. 2009, 2010c). We therefore interpreted the delay as an indication that the sex of the first egg was adjusted by selective abortion of follicles, as abortion would induce later laying due to additional time needed for maturation of a replacement follicle. Since abortion of follicles bears the cost of producing a follicle without benefits, perhaps pigeons apply this only for the most important and most frequently surviving chick: the one hatching from the first egg. Yet, whether pigeons are able to recruit a third follicle into the phase of rapid deposition of yolk was an important issue as recruitment of additional follicles is a prerequisite for the selective follicle-abortion hypothesis in order to avoid prolonged time intervals between oviposition of the eggs or the ovulation of premature follicles. Pigeons, however, are regarded as determinate layers and are thought to develop no more than two follicles per ovulation cycle (Haywood 1993). This assumption is supported by anatomical studies on closely related Ring Doves (*Streptopelia risoria*) (Cuthbert 1945) and Band-tailed Pigeons (*Columba fasciata*) (March and Sadleir 1970; Gutierrez et al. 1975) although it has been claimed that three mature follicles can occasionally occur simultaneously in domestic pigeons (Bartelmez 1912). However, even removal of eggs does not induce the production of additional eggs (Holmberg and Klint 1986; Blockstein 1989).

To test the follicle-abortion hypothesis we sacrificed female homing pigeons that had adjusted

primary sex ratio according to change in body mass (Goerlich et al. 2010a). We found no evidence for the hypothesis as autopsies neither revealed signs of abortion/regression nor additional mature pre-ovulatory follicles in the mothers. Including all autopsies performed during our studies of adjustment of primary sex ratio, we recorded a third follicle only in 6 out of 192 females. The low frequency of this phenomenon was in line with early findings by Bartelmez (1912) and severely undermines the follicle-abortion hypothesis.

Further analyses of the potential effects of testosterone on ovarian mass and morphology suggested as well that the delayed initiation of clutches was not due to increased incidents of follicular atresia or of suppressed ovulation (Goerlich et al. 2010b). Testosterone did not affect ovarian mass or the number of pre-hierarchical follicles. Also corticosterone delayed oviposition and biased sex ratio, but did not affect yolk mass (see above). Since both testosterone and glucocorticoids are known to have promoting as well as delaying effects on follicular maturation, follicular growth patterns would need to be analyzed in more detail to gain further insight (Badyaev et al. 2005; Drummond 2006; Tetsuka 2007).

Yolk hormones

Avian eggs, like eggs of other oviparous species, contain substantial amounts of androgens originating from the maternal system (Groothuis et al. 2005). One potential pathway for manipulation of the primary sex ratio is that yolk hormones affect the sex of the embryo, perhaps by meiotic drive. However, we did not find sex-specific differences in concentrations of testosterone either in the complete yolk or in the outer-layer (where the blastocyst is located), suggesting that in pigeons maternal testosterone does not result in a biased sex ratio via the yolk pathway (Goerlich et al. 2009, 2010c). Similarly, about 20 studies have investigated differences in yolk hormones according to the sex of the embryo, and these differences were only apparent based on *post-hoc* analyses in combination with other factors such as food quality or maternal social status (reviewed by Goerlich and Groothuis, in preparation). In addition, we did not find a relation between primary sex ratio and circulating plasma levels of either testosterone or corticosterone measured during the phase of rapid follicular growth, when maternal hormones are accumulating in the yolk (Goerlich et al. 2009, 2010c).

Although testosterone did not reduce maternal body condition or fecundity (Goerlich et al. 2009, 2010b, 2010c), the delayed initiation of clutches seems detrimental for maternal fitness. The results are relevant for the discussion of the benefits and costs of elevated testosterone in females (Ketterson et al. 2005). The detrimental effects of elevated plasma testosterone might require the breeding female to uncouple deposition of testosterone in the yolk from her own circulation in case elevated testosterone levels in the yolk would benefit her chick. This suggestion is currently heavily debated in the literature. Several studies have shown that implanting breeding females with hormones results in eggs with elevated concentrations of hormones, indicating that females cannot regulate concentration of yolk hormone independently from the concentrations in their own plasma. However, these studies have used hormone dosages resulting in supra-physiological plasma levels, potentially overflowing the maternal system (reviewed by Groothuis and Schwabl 2008). Contrarily, we used testosterone implants that elevated maternal plasma concentrations within the physiological range of the species and this did not affect the concentration in the total yolk, the outer-layer of yolk, or the yolk in follicles (Goerlich et al. 2009, 2010b, 2010c). Interestingly, testosterone concentrations in second, un-ovulated follicles were three times as high as in second eggs after oviposition, regardless of whether the latter were incubated for 3 days or were fresh (Goerlich et al. 2009, 2010b). This phenomenon was also shown in quail (Okuliarová et al. 2010) and House Sparrows (Egbert et al. 2013), although to a much lesser extent. While in quail, total yolk testosterone (concentration corrected for yolk mass) was similar between pre-ovulatory follicles (Okuliarová et al. 2010), in pigeons total testosterone remains significantly higher in pre-ovulatory follicles compared with yolks of freshly laid eggs (Goerlich, unpublished data). This suggest that testosterone and the dynamics of yolk accumulation during the last stage of follicular maturation differ between species, as suggested by Egbert et al. (2013) who showed that even late stage (F1) pre-ovulatory follicles enhance testosterone production in response to GnRH treatment of the female, while in domestic chickens testosterone production decreases in F1 follicles (Bahr et al. 1983). This poses the question of whether measuring yolk hormones after oviposition correctly reflects maternal allocation. Besides highlighting this methodological issue, these findings inspire further research into the change of yolk hormones not only over the course of incubation but also during the formation of eggs.

A role for prolactin?

The significant variation in primary sex ratio in relation to the order in which eggs are laid has

also been shown in several studies of the house finch (*Carpodacus mexicanus*) (e.g., Badyaev et al. 2005, 2006). The sex-specific patterns are correlated with the population-specific and individual-specific incubation behavior of the mother as clutches that are incubated from the first egg onwards show laying-order-specific deviations in the eggs' sex (especially the two eggs laid first), whereas this is absent in clutches being incubated only after completion of oviposition (Badyaev et al. 2003). The pituitary hormone prolactin regulates the onset of incubation. Induced by high and low ambient temperatures, prolactin levels can diverge between individuals and populations and lead to differences not only in incubation behavior. Mothers with high prolactin levels were more likely to produce female oocytes, while low prolactin levels were related to male oocytes (Badyaev et al. 2005). Interestingly, prolactin has also been related to body condition (Criscuolo et al. 2006), stress physiology (Angelier and Chastel 2009), and deposition of androgens to the yolk (Sockman et al. 2006), all of which have been suggested to affect sex ratio of the offspring (Pike and Petrie 2003; Alonso-Alvarez 2006). Moreover, prolactin stimulates the production of crop milk in Columbidae and rises markedly during incubation (Horseman and Buntin 1995). Finally, prolactin is involved in oocyte development and in timing of meiosis, at least in mammals (Torner et al. 2001).

The potential role of maternal prolactin in maternal manipulation of sex ratio is promising and might explain both the seasonal shift of sex ratio we found under natural conditions (Dijkstra et al. 2010) and the sequence-specific sex bias in pigeons (Goerlich et al. 2009, 2010c). First, prolactin levels, like the primary sex ratio, vary across the season and usually increase late in the breeding season (reviewed by Sockman et al. 2006; pigeons: Saarela et al. 1986), the time when we found first eggs to be female biased (Dijkstra et al. 2010). Second, the seasonal change in prolactin may adjust incubation behavior according to changes in ambient temperature, as suggested for the house finch. In conditions that are either too cold or warm, an earlier rise in prolactin would stimulate an earlier start of incubation during clutch production, thereby protecting the first egg's viability. The variation in onset of incubation and hormonal condition of the mother would have the greatest influence on the first egg to be laid, as incubation always starts immediately after the second egg is laid, but varies in relation to oviposition of the first egg. Therefore, meiotic drive influenced by maternal hormone levels (e.g., prolactin) has much more potential in the variable environment during meiosis of the first follicle than in the constant milieu during meiosis of the second follicle. Manipulation of maternal prolactin levels, ideally at the time of meiosis, might provide some more insight.

Conclusion

Both under natural changing conditions and in response to experimental manipulations, pigeons show remarkable consistent evidence of biases in sex ratio that are evident in first eggs but not in second eggs. First eggs early in spring are male biased, while in autumn first eggs are female biased (Dijkstra et al. 2010). Removal of eggs in combination with restriction of food results in a female bias of first eggs (Pike 2005), whereas an increase in body mass induces a male bias (Goerlich et al. 2010c). Elevation of testosterone in females induces male bias of first eggs if maternal body mass remains stable (Goerlich et al. 2009). Elevation of corticosterone induces a female bias of first eggs, at the same time uncoupling the relationship with maternal body condition.

Based on our studies on Homing Pigeons we can exclude the hypothesis of sex-specific fertilization and zygote formation, by comparing the proportions of infertile eggs (Pike 2005; Goerlich et al. 2009, 2010c; Dijkstra et al. 2010). By performing autopsies we are able to reject the follicle-abortion hypothesis as we found very few additional follicles and no aborted follicles (Goerlich et al. 2010a). Based on our data and on the literature it is unlikely that the sex bias was induced via hormones in the yolk (Goerlich et al. 2009, 2010c). The delayed initiation of the clutches supports the view that the pattern of follicular maturation might have changed (Goerlich et al. 2009, 2010c), potentially affecting sex determination. Finally, we showed the importance of the level of corticosterone in the maternal plasma at about the time of meiosis, possibly influencing hormone segregation. Unfortunately, we did not measure plasma testosterone at meiosis; still, these results strongly suggest that the maternal hormonal state, specifically the time of meiosis, influences sex determination, further suggesting that meiotic drive is involved in biases in primary sex ratio in pigeons.

The next step should be to test the relationship between temperature, prolactin, start of incubation, and primary sex ratio in pigeons, ideally by applying the same techniques as Badyaev et al. This should also include description of follicular growth patterns and measurements of hormone levels in the yolk. Given the change in yolk testosterone across formation of the follicle and incubation, concentrations should be measured as soon after meiosis as sufficient embryonic tissue for DNA extraction is

available (e.g., in freshly laid eggs) (Aslam et al. 2012). Because of the importance of maternal condition, measurements of body mass should be taken regularly. Furthermore, steroids such as progesterone, which are involved in incubation behavior in pigeons (reviewed by Murton and Westwood 1977, p. 123) and manipulation of primary sex ratio (Correa et al. 2005), could be of interest as well. Finally, there has not been much research on characterization of steroid receptors on the oocyte membrane and to our knowledge only the androgen receptor has been described in follicles at different stages of maturation (Yoshimura et al. 1993). Nevertheless, receptor densities might be crucial for the regulation of steroid uptake into the yolk. *In vitro* culture of follicles and ovaries would be very useful to further explore molecular aspects and the effects of steroid hormones on follicular development and meiosis.

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